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Detection of Clonal Histiocytes in Langerhans Cell Histiocytosis: Biology and Clinical Significance

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Summary Although the first clinical description of Langerhans cell histiocytosis (LCH) was published over a century ago, the aetiology and pathogenesis of this enigmatic disorder are still remained unknown. Viral, immunological, neoplastic and other pathogenetic mechanisms have been considered, but none has been proven. The prevailing opinion is that LCH is a reactive disorder rather than a neoplastic process, but this presumption has never been definitively tested. A key feature of a neoplasm is its clonal derivation from a single cell. To determine if LCH is a polyclonal reactive or a clonal disorder, we and others have recently used molecular biological techniques to assess clonality in LCH. Using X chromosome-linked DNA probes that can detect clonal or polyclonal X chromosome inactivation patterns in female tissues, clonal CD1a+ histiocytes have now been detected in the lesional tissues in each of 16 females affected with LCH. Most of these patients were studied prior to the initiation of therapy. Lymphoid clonality was excluded in all cases in which it was studied, confirming that the clonal cells in LCH are the CD1a+ dendritic cells presumed to be pivotal in this disorder. Two distinct lesions (a pre-treatment bone biopsy and a lymph node biopsied 3 years later) have been studied in only one case to date; the same clonal pattern of X chromosome inactivation was observed, consistent with persistence of the same clone during this patient's disease course. Unexpectedly, clonal histiocytes were detected in all forms of LCH (in infants with "disseminated disease" involving lymph nodes, liver, spleen, and bone marrow; in females with multisystem disease involving skin, bones, and other organs; and in females with unifocal bone disease). Although the biological significance of the detection of clonality in the histiocytic lineage is not yet fully understood, these data suggest that LCH may be a clonal neoplastic disorder with highly variable biological behaviour and clinical severity. As all forms of LCH appear to be clonal, assessment of clonality at diagnosis will not be useful in predicting outcome in individual patients. However, this discovery provides clear directions for studies that must now be undertaken to understand more fully the biological and clinical significance of clonality in LCH.

Introduction

The clinical scope of Langerhans cell histiocytosis is wide, ranging from a potentially lethal leukemia-like process that primarily affects infants to a solitary lytic lesion of bone that is usually cured by simple curettage. Intermediate forms of disease, characterized by lesions of bone, skin, and mucous membranes, are associated with varying degrees of organ dysfunction, diabetes insipidus, and a chronic indolent course. In contrast to this clinical heterogeneity, the pathologic lesions in all forms of LCH are more uniform and feature CD1a+ histiocytes ('LCH cells') with a morphology and phenotype similar to that of dendritic antigen-presenting Langerhans cells found in the skin and other organs (Nezelof *et al.*, 1973, Favara, 1991).

Criteria for definitive diagnosis and clinical classification of LCH (Writing Group of the Histiocyte Society, 1987) have now been firmly established but the aetiology and pathogenesis of this disorder are still poorly understood. Although a viral aetiology has been seriously considered, recent studies have failed to detect EBV, HHV6, and seven other viral genomes in LCH tissues (McClain *et al.*, 1994 and this volume, Mierau *et al.*, 1994). The high probability of survival in LCH, particularly in patients greater than two years of age without organ dysfunction (Greenberger *et al.*, 1981; Komp, 1987, 1991; McLelland *et al.*, 1990), the occurrence of rare spontaneous remissions (Broadbent *et al.*, 1984), the failure to detect aneuploidy in LCH lesions using flow cytometric techniques (Rabkin *et al.*, 1988; McLelland *et al.*, 1989), and the failure to obtain metaphases and detect karyotypic abnormalities in lesional cells have all contributed to the current opinion that LCH is a reactive disorder of immune regulation and not a neoplastic process. However, this presumption has not been definitively tested.

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A key feature of a neoplasm is its clonal origin of derivation from a single cell (Knudsen, 1985). Clonality can now be assessed in any cell lineage in the majority of females by molecular analysis of patterns of X chromosome inactivation (for review see Vogelstein *et al.*, 1985, 1987; Busque & Gilliland, 1993). These new molecular assays are extremely powerful, particularly when coupled with polymerase chain reaction (PCR) techniques which allow analysis of small tissue samples or small numbers of cells, with phosphorimager quantitation which allows for precise detection of small numbers of clonal cells, and with flow cytometric sorting which allows clonality to be determined in selected cell populations. As reviewed in this chapter, the application of these assays to LCH lesions has revealed that all forms of LCH are clonal (Willman *et al.*, 1993a, 1993b, 1994; Yu *et al.*, 1994).

Background and methods

Molecular assays for the assessment of clonality are predicated upon the random process of X chromosome inactivation in females (the "Lyon Hypothesis"). In females, one X chromosome is randomly inactivated in each somatic cell early in embryogenesis; this pattern of inactivation is stably transmitted to all progeny cells and persists during malignant transformation (Wainscoat & Fey, 1990). To assess clonality, DNA probes are first used to detect polymorphisms at a particular X-linked locus, so that the maternal and paternally-derived X chromosomes can be distinguished. Secondly, differential methylation or gene expression is used to distinguish the active from the inactive X chromosome. Ideally, in a polyclonal tissue, half of the cells will have inactivated the paternally- derived and half the maternally-derived X chromosome. In a monoclonal or 'clonal' population of cells, by contrast, one X chromosome will be exclusively inactivated (see Figure 1).

For an X-linked DNA locus to be useful in the assessment of clonality, two criteria must be met: (a) the locus must have

a high degree of "informativeness" (i.e., a high frequency of polymorphisms at this site in the population) so that the paternal and maternal X chromosomes can be distinguished in most individuals; and, (b) the inactive (or active depending upon the locus) X chromosome must be consistently methylated in different tissues so that the activation status of the paternal and maternal chromosomes can be reliably distinguished. Recent studies have revealed that up to 25% of females may have non-random patterns of X chromosome inactivation (skewed Lyonization) in their normal tissues which may mimic clonal derivation of cells (Gale *et al.*, 1991); thus, another requirement for clonality analysis is that the X chromosome inactivation patterns must be compared, in each individual, in lesional and normal cells derived from a similar embryological lineage. Three X-linked polymorphic DNA probes [phosphoglycerate kinase (PGK), hypoxanthine phosphoribosyl transferase (HPRT), and the hypervariable locus DXS255 (M27b)] were initially used for clonality analysis; however, the use of these probes was limited either by a low degree of informativeness or by variable methylation (reviewed in Busque & Gilliland, 1993). These technical problems, as well as a relative lack of sensitivity, limited the conclusions that we could draw in our initial clonality studies in LCH (Willman *et al.*, 1993a, 1993b). However, these limitations have recently been overcome by using the X-linked human androgen receptor (*HUMARA*) gene (Allen *et al.*, 1992). Analysis of clonality at the *HUMARA* locus is superior because of its high rate of informativeness (>85% of individuals are heterozygous for the CAG repeat length at this locus, as discussed below and in Figure 1) and a consistent pattern of methylation (Busque & Gilliland, 1993; Busque *et al.*, 1994; Willman *et al.*, 1994).

A schematic representation of the HUMARA clonality assay is provided in Figure 1. Paternal and maternally-derived X chromosomes can usually be distinguished at the *HUMARA* locus because the number of repeats of a tandemly-repeated triplet nucleotide DNA sequence (CAG) is highly variable (i.e. polymorphic) in our population. Variations in the length of the CAG repeat on the paternal and the maternal X chromosomes will yield *HUMARA* alleles of different lengths, thereby allowing the two X chromosomes to be distinguished (Figure 1). The methylation-sensitive restriction enzyme sites HpaII and HhaI, immediately upstream of the CAG repeat (Figure 1A), are then exploited to determine whether the paternally or the maternally-derived chromosome, or both, are active in a particular tissue; at the HUMARA locus, the active (expressed) X chromosome is non-methylated while the inactive chromosome is methylated. To perform the HUMARA assay (see Willman *et al.*, 1994 for a detailed review), DNA is isolated from a tissue or cells of interest and is then digested with the methylation-sensitive enzymes HpaII or HhaI, which cleave the restriction sites only on the active (nonmethylated) chromosomes. When this restricted DNA is subjected to PCR amplification, only the methylated (inactive) *HUMARA* alleles are amplified (Figure 1B); the PCR cannot amplify active (digested) *HUMARA* alleles because the Taq DNA polymerase used for PCR amplification cannot extend beyond the cleaved DNA strand. In a polyclonal population, random X chromosome inactivation results in the methylation of either the maternal or the paternal chromosomes in different cells; in this setting PCR will amplify both the methylated (inactive) paternal and maternal X chromosomes. Since they are of different lengths (Figure 1B) these different X chromosome *HUMARA* alleles may then be resolved with gel electrophoresis. In contrast, in a clonal population with nonrandom X inactivation, only one *HUMARA* allele is methylated (in this example – Figure 1B – the longer, maternal allele) and will be amplified.

A representative *HUMARA* assay performed on DNA isolated from a lymph node that was virtually effaced by CD1a+ Langerhans cells is shown in Figure 2. Two HUMARA alleles (one from the maternal and one from the paternal X chromosome) were obtained when DNA was amplified in the PCR without first being digested with HpaII; the detection of two differently-sized alleles means that this

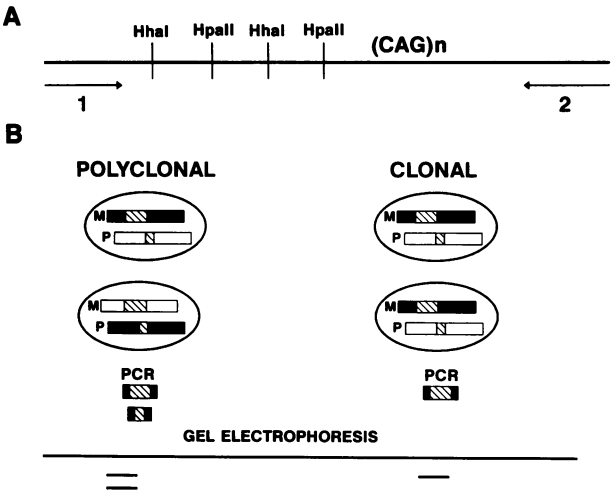

Figure 1 The HUMARA clonality assay. (**A**) Map of the *HUMARA* (human androgen receptor gene) locus. Methylation-sensitive restriction enzyme sites (HpaII and HhaI) are located 100 base pairs 5′ of the polymorphic CAG repeat [$(CAG)_n$] in the first exon of the *HUMARA* gene. Radioactively-labelled oligonucleotide primers (arrows labelled 1 and 2) flanking the methylation sites and the repeat region are used to amplify the polymorphic CAG repeat expansion in exon 1 of the *HUMARA* locus in the PCR. (**B**) Expected HUMARA Results in Polyclonal or Monoclonal Cells. In this example, the maternal X chromosome (M) has a longer CAG repeat at the *HUMARA* locus (hatched box) than the paternal X chromosome (P). Methylated chromosomes are shown in black. In a polyclonal cell population (left), a random pattern of X chromosome inactivation results in methylation of either the paternal or the maternal X chromosome in each individual cell. In contrast, in a clonal cell population (right), only one X chromosome will be consistently methylated (inactivated); in this example, the maternal (M) chromosome with the longer CAG repeat is consistently methylated. As discussed in the text, only methylated *HUMARA* alleles are amplified in the PCR. Thus, in a polyclonal population, both maternal and paternal alleles will be amplified and these alleles can then be distinguished in gel electrophoresis because they are of different lengths. In contrast, in a clonal population, only one allele will be selectively amplified.

patient is informative at the *HUMARA* locus. However, when DNA was first pre-cut with HpaII and then amplified in the PCR, the lower allele with the shorter CAG repeat was preferentially amplified; virtually no signal was obtained from the upper allele. These results are due to the presence of a predominant clonal population of cells.

Molecular assessment of clonality in LCH

The molecular assessment of clonality can be problematic in cytologically heterogeneous lesions, such as LCH, or in tissues in which clonal cells are admixed with reactive polyclonal cells. As discussed below, two modifications to the HUMARA assay have been developed recently to overcome these difficulties. To date, modified HUMARA assays have been performed by four different research groups on DNA isolated from lesional tissues, mostly obtained prior to treatment, in 16 females affected by LCH (Table 1). In all cases, the CD1a+ cells were determined to be clonal (Table 1).

To improve the sensitivity of the HUMARA assay to detect clonal cells admixed with polyclonal cells, we modified the HUMARA PCR procedure and analyzed the radioactive content of the resultant PCR products in gels with highly sensitive phosphorimager analysis (Busque & Gilliland, 1993; Willman *et al.*, 1993b, 1994). Standard curves were established using mixtures of polyclonal and known clonal cells; using these curves the quantitative data could be converted to a percentage of clonal cells (Willman *et al.*, 1994). With these modifications, the HUMARA assay could then con-

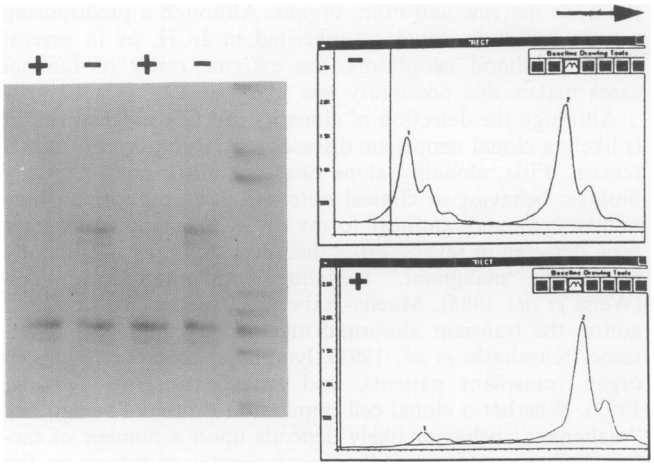

Figure 2 HUMARA clonality assay in lymph node affected by LCH. (Left panel). Autoradiograph of electrophoresed HUMARA PCR products. DNA isolated from a lymph node virtually effaced by CD1a+ cells was either pre-digested with HpaII (+) or was undigested (−) and then amplified in the HUMARA PCR assay as described (Willman *et al.*, 1994). PCR products were electrophoresed and autoradiographs obtained. Duplicate samples are shown. With pre-digestion (+), only the lower HUMARA allele is amplified, consistent with clonality. In contrast, in undigested DNA both alleles are amplified. As shown in the autoradiographs, amplification of each allele actually yields a major band and a minor (slightly faster migrating) shadow band; this shadow band results from slippage of the DNA polymerase during PCR amplification of the repeat expansion. (Right Panel). Phosphorimager quantitation of HUMARA PCR products. In the analysis of a mixed population of polyclonal and clonal cells, the relative intensity of each *HUMARA* allele is proportional to the percentage of clonal cells present in the polyclonal population. To make this assay highly sensitive, the radioactive content of each PCR product may be precisely quantitated with a phosphorimager (see Willman *et al.*, 1994). The scan obtained by imaging the PCR products obtained from undigested DNA (upper panel; (−)) reveals two alleles of relatively equal intensity. In contrast, in pre-digested DNA (lower panel; (+)), virtually all radioactivity is contained in the lower allele.

sistently and sensitively detect a clonal cell population if it comprised at least 10% of the cells in a polyclonal background. Using this approach, we studied matched lesional tissues and paired leukocyte controls in 10 previously untreated females with various forms of LCH: 3 females (3 months, 4 months, and 3 years of age) with "acute disseminated LCH" (as defined by Nezelof *et al.*, 1979) involving skin, liver, spleen, and lymph nodes; four females (13 months, 24 months, 4 years, and 25 years of age) with the intermediate, multisystem form of LCH with multiple bone lesions and varying degrees of organ dysfunction; and, 3 females (6, 4, and 14 years of age) with unifocal bone disease ('eosinophilic granuloma'). In one case (the 25 year old female with multisystem disease) clonality could not be determined due to extreme constitutional lyonization, but a variable percentage of clonal cells was detected in each of the remaining 9 cases. In each case, moreover, the percentage of clonal cells determined by the HUMARA assay correlated closely with the percentage of CD1a+ cells in the lesion. This close correlation, along with the absence of a clonal lymphoid population in each case, strongly suggested that the clonal cells in LCH are the CD1a+ cells presumed to be the pivotal cell in this disease. Recently, Arceci, Busque, and Gilliland (Dana-Farber Cancer Institute, Boston, MA, USA; personal communication) also applied this modified HUMARA assay to a skin biopsy obtained from a young female with disseminated LCH; interestingly, this affected child had an unaffected identical twin. HUMARA assays revealed a clonal population similar in percentage to the CD1a+ cells in the lesion (Table 1).

In an elegant modification to the HUMARA assay, Yu *et al.*, 1994 used flow cytometric sorting to purify CD1a+ cells from the lesional tissues of 3 previously untreated females affected with multisystem LCH. CD1a+ cells were sorted from an affected lymph node in a 6 month old female, affected bone marrow in a 20 month old female, and the subcutaneous tissue of a 4 year old female. DNA was then extracted from the sorted CD1a+ cells and the HUMARA assay was performed as described (Yu *et al.*, 1994). In each case, the sorted CD1a− population was essentially "purely" clonal, while a sorted control population of CD1a-negative cells from the same tissue was polyclonal. This same approach was also recently used by Kannourakis and Tiong (Royal Childrens Hospital, Victoria, Australia) on three females (treatment status and age unknown) with LCH; again, in each case the sorted CD1a+ cells were clonal (Table 1, personal communication; initial data presented at the 9th Annual Meeting of the Histiocyte Society in San Francisco, CA, October, 1993). Thus, flow cytometric sorting coupled with the HUMARA PCR approach has provided definitive proof that the CD1a+ Langerhans cells are the clonal cells in LCH.

In summary, the lesional tissues of 16/16 females affected by LCH have now been determined to contain clonal CD1a+ cells. Clonal CD1a+ cells have been detected in all clinicoanatomical forms of LCH and in several different types of tissues that are frequently involved by LCH (lymph node, bone marrow, bone, and skin). Of remaining interest is whether the same clone "persists" during the course of disease in each patient, particularly in those patients who have a protracted, chronic course. We have been able to address this issue in one of our 10 cases from whom tissues for clonality analysis were generously provided by Dr. Ronald Jaffe (Childrens Hospital, Pittsburgh, PA, USA). This female had a rash as a neonate then presented at 2 years of age with a temporal bone lesion and diabetes insipidus. Biopsy of the mastoid bone confirmed a diagnosis of LCH and retrospective study of this frozen material revealed clonal cells using the HUMARA assay (Willman *et al.*, 1994). She was treated with prednisone and vinblastine but 10 months after diagnosis developed rapid weight gain and a large hypothalamic lesion. The brain lesion was irradiated and the lesions of the mandible and maxilla were treated surgically. Despite continued chemotherapy, prominent cervical lym-

Table 1 Molecular analysis of clonality in LCH

No. cases studied	*Extent of Disease*	*Tissue examined*	*HUMAR modification*	*Results*	*Reference*
3	"Disseminated" Multisystem	LN	Quantitiation	3/3 Clonal	Willman *et al.*, 1994
3	Multifocal Bone/Skin/CNS	Bone	Quantitiation	3/3 Clonal	Willman *et al.*, 1994
3	Unifocal Bone	Bone	Quantitiation	3/3 Clonal	Willman *et al.*, 1994
1	"Disseminated" Multisystem	Skin	Quantitiation	1/1 Clonal	Arceci *et al.*, (Unpublished)
1	Multisystem	LN	CD1a+Sorting	1/1 Clonal	Yu *et al.*, 1994
1	Multisystem	BM	CD1a+Sorting	1/1 Clonal	Yu *et al.*, 1994
1	Multisystem	Soft Tissue	CD1a+Sorting	1/1 Clonal	Yu *et al.*, 1994
2	Multisystem	LN	CD1a+Sorting	2/2 Clonal	Kannourakis and Tiong (Unpublished)
1	NA	Bone	CD1a+Sorting	1/1 Clonal	Kannourakis and Tiong (Unpublished)

Abbreviations: N.A., (Information) Not Available; LN, lymph node; BM, bone marrow.

phadenopathy developed 23 months later. Biopsy of this node confirmed the diagnosis of LCH and clonal cells were detected in the HUMARA assay; importantly, this second clonality study detected a clone which had inactivated the same HUMARA allele as the clone from the initial diagnostic bone biopsy (Willman *et al.*, 1994). These studies do not definitively prove that the two clones were the same, but the results are consistent with persistence of the same clone during this patient's disease course. Clearly, sequential clonality studies should be performed on more LCH patients with various forms of disease.

Discussion and future directions

The molecular approach for the assessment of clonality has definitively demonstrated that LCH is a clonal histiocytic disease, rather than a reactive polyclonal disorder. To our group of investigators, the discovery that all forms of LCH are clonal was unexpected. The detection of clonal histiocytes in children affected with the acute disseminated form of disease was not as surprising because this "leukaemia-like" form of LCH often behaves like a malignant neoplastic process with mortality rates approaching 60–65% in infants (Lahey, 1962; Nezelof *et al.*, 1979; Greenberger *et al.*, 1981; Komp, 1987). In contrast, the finding of clonal histiocytes in children with intermediate, multisystem disease was more surprising as this form has a 60–70% survival rate[1] in children less than two years and an 80% survival rate in children greater than two years of age (Lahey, 1962; Greenberger *et al.*, 1981; Komp, 1987); spontaneous regression may also rarely occur (Broadbent *et al.*, 1984). The finding of clonal histiocytes in unifocal LCH was completely unexpected as this form of disease is the most clinically benign.

The high probability of survival in LCH and the reported spontaneous regressions require that the clinical and biological significance of the detection of clonality in all forms of LCH be carefully considered. It is now widely agreed that most neoplasms are clonal, consistent with the somatic mutation theory of carcinogenesis which assumes that a neoplasm results from the progeny of a single cell that has acquired one or more somatic mutations (Knudsen, 1985, Wainscoat & Fey 1990). In our opinion, the discovery that all forms of LCH are clonal implies that LCH is most likely a clonal neoplastic disease with highly variable biologic behavior and clinical severity. From this perspective, LCH must arise from acquired somatic genetic mutations that give rise to the clonal expansion of Langerhans cells or their precursors in the bone marrow and other organs. Although a predisposing genetic mutation could be inherited in LCH, as in several other childhood neoplasms, the extreme rarity of familial cases makes this possibility less likely.

Although the detection of clonality in LCH suggests that it is likely a clonal neoplastic disease, several important caveats remain. First, clonality alone does not allow one to predict biologic behavior or clinical outcome. Like the intermediate multisystem and unifocal forms of LCH, clonal cells have been detected in several other disorders that may not initially be overtly "malignant," including lymphomatoid papulosis (Weiss *et al.*, 1986), Mucha-Haberman disease, multinodular goitre, the transient abnormal myelopoiesis of Down Syndrome (Kurahashi *et al.*, 1991), lymphoproliferative lesions in organ transplant patients, and aplastic anaemia (Young, 1992). Whether a clonal cell population exhibits "benign" or "malignant" behavior likely depends upon a number of factors including the constellation of genetic mutations in the clone, the tissue microenvironment, and an individual's host response to clonal cells. Secondly, the definitive proof of the neoplastic aetiology of LCH must await the identification of potential somatic genetic mutations, as discussed below. Finally, more must be understood of the normal, presumed polyclonal, expansion of histiocytes and dendritic cells from bone marrow precursors. In aplastic anaemia, haematopoietic cell clonality or oligoclonality may arise because the stem cell pool is limited (Young, 1992). Although it is unlikely that such a situation exists in LCH, more must be understood of the development of dendritic cells from bone marrow precursors and their subsequent differentiation/activation at distant body sites.

The discovery of clonality in LCH does point to immediate research directions. Clonality should be assessed sequentially in more LCH patients at the time of disease presentation and during the course of disease. In this way, we will determine whether the same clone persists in each individual. The search for cytogenetic and molecular genetic mutations in LCH lesions must begin anew. Although traditional cytogenetic approaches have not been successful in LCH, contemporary cytogenetic methods, employing haemopoietic growth factors and molecular techniques such as fluorescence *in situ* chromosomal hybridization (FISH), and comparative and subtractive genomic hybridization hold promise for the future. The molecular assessment of clonality in other histiocytic disorders, such as juvenile xanthogranuloma and sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease), is also now indicated. Further biological studies of the development and homing of dendritic cells, and the interaction between the immune system and clonal CD1a+ cells will provide a biological framework for the interpretation of new scientific studies in LCH.

[1]The survival rate in these groups is even higher than these figures indicate; Eds

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